Am. J. Trop. Med. Hyg., 31(1), 1982, pp. 149–155

STUDIES ON THE ANTIGENIC RELATIONSHIP AMONG PHLEBOVIRUSES*

ROBERT B. TESH, CLARENCE J. PETERS, AND JAMES M. MEEGAN

Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine, 60 College Street, Box 3333, New Haven, Connecticut 06510, and U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

Abstract. The antigenic relationship of the 31 currently recognized phlebotomus fever group viruses was examined by plaque reduction neutralization test. Although some low-level cross-neutralization was observed, each of the viruses was easily differentiated by this method. Rift Valley fever virus was shown to be antigenically related to Candiru, Frijoles, Karimabad and Punta Toro viruses. Naples, Tehran and Toscana viruses were also shown to be closely related. Preliminary results of indirect fluorescent antibody tests indicate that this technique is broadly reacting and less specific than either the complement-fixation or neutralization test for identifying phlebotomus fever group viruses. A discussion follows on some of the difficulties encountered in attempting to classify phleboviruses.

Recently published reports[1,2] that Rift Valley fever (RFV) virus is antigenically and biochemically related to several viruses in the phlebotomus fever group (family Bunyaviridae, genus *Phlebovirus*) have renewed interest in this little studied group of arthropod-borne viruses. The phlebotomus fever (PF) group currently consists of 31 district virus serotypes (Table 1). These agents occur in both the Old and New Worlds, and each serotype appears to have a different geographic distribution.[3] In general their natural history is poorly understood. The majority have been associated with phlebotomine sandflies,[3,4] but at least three of the serotypes (Arumowot, Itaporanga and Rift Valley fever) appear to be mosquito-borne.[3,5] Seven PF group viruses (Alenquer, Candiru, Chagres, Naples, Punta Toro, Rift Valley fever and Sicilian) have been isolated from sick persons.[3,6,7] Three of these agents (Naples, Sicilian and Rift Valley fever) are of proven public health importance. In addition, serologic evidence of human infection has been demonstrated with seven other PF group viruses (Arumowot, Bujaru, Gabek Forest, Gordil, Karimabad, Saint Floris and Toscana).[3,8,9]

In 1975, Tesh et al.[3] described the antigenic relationship of 22 PF serotypes known at that time. By hemagglutination-inhibition (HI) test, the viruses showed considerable cross-reactivity.[3] Results of complement-fixation (CF) and plaque reduction neutralization (PRN) tests were more specific, however, allowing clear separation of the various serotypes.[3] Since publication of the aforementioned data, nine additional virus serotypes have been included in the PF group. In order to update these earlier serologic studies and to determine the antigenic relationship of RVF virus, cross-neutralization tests were performed on the 31 currently recognized *Phlebovirus* serotypes. The present paper summarizes this work and also describes preliminary studies on the use of immunofluorescence to identify PF group viruses and antibodies.

Accepted 29 May 1981.

* This work was supported in part by research contracts NO1 AI 82560 from the National Institute of Allergy and Infectious Diseases, DAMD 17-80-C-0178 from the U.S. Army Medical Research and Development Command, and DOD contract N00014-78C-0104. Portions of this work were presented at the Workshop on Rift Valley Fever, March 18–20, 1980, in Herzlia, Israel.

MATERIALS AND METHODS

Viruses

The identity of the 31 PF viruses used in this study is given in Table 1. The RVF virus stock used in PRN tests was prepared from infected fetal rhesus monkey lung 103 cells. All of the other virus stocks were prepared from infected Vero cells.

TABLE 1

Viruses and immune reagents used in neutralization tests

Virus serotype	Strain	Source of immune reagents*
Aguacate	VP-175A	HS(3)
Alenquer	Be H 301101	GPS(3)
Anhanga	Be An 46852	GPS(3)
Arumowot	Ar 1284-64	HS(3)
Buenaventura	Co Ar 3319	HS(3)
Bujaru	Be An 47693	GPS(3)
Cacao	VP-437R	HS(3)
Caimito	VP-488A	HS(3)
Candiru	Be H 22511	MAF(1)
Chagres	JW 10	MAF(2)
Chilibre	VP-118D	HS(3)
Frijoles	VP-161A	HS(3)
Gabek Forest	Sud An 754-61	RS(3)
Gordil	Dak An B 496d	MAF(4)
Icoaraci	Be An 24262	HS(3)
Itaituba	Be An 213452	GPS(3)
Itaporanga	Original	HS(3)
Karimabad	I-58	MAF(3)
Naples (Sandfly fever)	Naples	MAF(3)
Nique	Nique-9C	HS(3)
Pacui	Be An 27326	HS(3)
Punta Toro	D-40210 A	MAF(1)
Rift Valley fever	Zagazig 501† (Entebbe)	RB(1)
Rio Grande	TBM4-719	MAF(?)
Saint Floris	DAK An B 512	GPS(3)
Salehabad	I-81	MAF(3)
Sicilian (Sandfly fever)	Sicilian	MAF(3)
Tehran	I-47	GPS(3)
Toscana	ISS. Phl. 3	MAF(?)
Turuna	Ar 352492	MAF(3)
Urucuri	Be An 100049	HS(3)

* HS = hamster serum; GPS = guinea pig serum; RB = rabbit serum; RS = rat (*Rattus norvegicus*) serum; MAF = mouse ascitic fluid. Number in parentheses refers to the number of injections of antigen which the animal received.

† Zagazig 501 strain used in neutralization tests and Entebbe strain used to prepare the immune serum.

Immune reagents

Immune sera and ascitic fluids against each of the PF serotypes were prepared in rodents or rabbits. The donor species as well as the immunization schedule for the immune reagents are listed in Table 1. Most of the animals received three intraperitoneal injections of viral antigen at 14- to 21-day intervals. Freund's complete adjuvant was added to the second and third injections. Immunized mice were inoculated with sarcoma 180 cells 1 week after the third injection to induce formation of ascitic fluid.

Neutralization test

Neutralization tests were performed by a previously described plaque technique[3] in microplate cultures of Vero cells, using a constant virus inoculum (40–100 plaque forming units) against varying dilutions of the immune reagents. The latter were prepared in twofold dilutions from 1:10 to 1:20, 480 in phosphate-buffered saline, pH 7.2, containing 0.5% gelatin. After addition of virus to the diluted immune reagents, the resulting mixture was incubated overnight at 4°C prior to inoculation. Two microplate wells were inoculated with each dilution. Those specimens producing ≥90% plaque reduction were recorded as positive.

Complement-fixation test

Complement-fixation (CF) tests were done in the microtiter system,[10] using two full units of guinea pig complement. Viral antigens (10% infected newborn mouse brain) and immune ascitic fluids were tested in block titrations beginning at dilutions of 1:4.

Indirect fluorescent antibody test

The indirect fluorescent antibody (IFA) test was performed following procedures described previously by Riggs.[11] Vero cells, harvested 48 hours after infection (m.o.i. = 5–10), were used as antigen. The resultant cell harvest was mixed with an equal number of uninfected cells which served as controls. This cell mixture was placed in drops on Teflon-imprinted glass slides (Cel-line Associates),[11] air dried, and fixed in acetone. Immune reagents were prepared in serial twofold dilutions from 1:4 to 1:2,048 in phosphate buffered saline. After addition of the diluted immune reagents, antigen slides were incubated at 37°C for 60 minutes. Fluorescein-conjugated anti-mouse or anti-rabbit immunoglobulin-G, produced in goats, was used to strain the cells. All IFA tests were repeated at least twice for confirmation.

RESULTS

Table 2 summarizes PRN test results comparing 31 phleboviruses and their immune reagents. In general, the results were quite specific and each

TABLE 2

Results of plaque reduction neutralization tests with 31 phlebotomus fever group viruses

Immune reagent	Virus type*													
	AGU	ALE	ANH	AMT	BUE	BUJ	CAC	CAI	CDU	CHG	CHI	FRI	GF	GOR
AGUACATE	1,280†	0	0	0	0	0	0	0	0	0	0	0	0	0
ALENQUER	0	80	0	0	0	0	0	0	0	0	0	0	0	0
ANHANGA	0	0	320	0	0	0	0	0	0	0	0	0	0	0
ARUMOWOT	0	0	0	10,240	0	0	0	0	0	0	0	20	0	0
BUENAVENTURA	0	0	0	0	40	0	0	0	0	0	0	0	0	0
BUJARU	0	0	0	0	0	20	0	0	0	0	0	0	0	0
CACAO	0	0	0	0	0	0	80	0	0	0	0	0	0	0
CAIMITO	0	10	0	0	0	0	0	40	0	0	0	0	0	0
CANDIRU	0	0	0	0	0	0	0	0	320	0	0	0	0	0
CHAGRES	0	0	10	10	0	0	80	0	0	2,560	0	40	0	0
CHILIBRE	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	1,280	<20	<20	<20
FRIJOLES	0	0	0	0	0	0	0	0	0	0	0	10,240	0	0
GABEK FOREST	0	0	0	0	0	0	0	0	0	0	0	0	320	0
GORDIL	0	0	0	0	0	0	20	0	0	0	0	0	0	10,240
ICOARACI	0	10	0	40	10	0	20	0	20	0	0	20	0	0
ITAITUBA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ITAPORANGA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KARIMABAD	0	0	0	0	20	0	0	0	0	0	0	40	20	0
NAPLES	0	0	0	0	0	0	0	0	0	0	0	40	0	0
NIQUE	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PACUI	0	0	0	0	0	0	0	0	0	0	20	0	0	0
PUNTA TORO	0	0	10	0	40	0	10	0	40	0	0	320	0	0
RIFT VALLEY FEVER	0	0	0	0	0	0	0	0	0	0	0	20	0	0
RIO GRANDE	0	0	40	0	0	0	0	0	0	0	0	20	0	0
SAINT FLORIS	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SALEHABAD	0	0	0	40	0	0	—	0	10	0	0	80	0	0
SICILIAN	0	0	0	0	0	0	0	0	0	0	0	160	0	0
TEHRAN	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOSCANA	0	0	0	0	0	0	0	0	0	0	0	40	0	0
TURUNA	—	—	—	—	—	—	—	—	—	—	—	—	—	—
URUCURI	0	0	0	0	0	0	0	0	0	0	0	20	0	0

* AGU, Aguacate; ALE, Alenquer; ANH, Anhanga; AMT, Arumowot; BUE, Buenaventura; BUJ, Bujaru; CAC, Cacao; CAI, Caimito; CDU, Candiru; CHG, Chagres; CHV, Charleville; CHI, Chilibre; FRI, Frijoles; GOR, Gordil; GF, Gabek Forest; ICO, Icoaraci; ITA, Itaituba; ITP, Itaporanga; KAR, Karimibad; SFN, Naples; NIQ, Nique; PAC, Pacui; PT, Punta Toro; RVF, Rift Valley fever; RG, Rio Grande; SAF, Saint Floris; SAL, Salehabad; SFS, Sicilian; TER, Tehran; TOS, Toscana; TUA, Turuana; URU, Urucuri.

† Reciprocal of highest antiserum or ascitic fluid dilution producting >90% plaque reduction. 0 = <1:10. — = not tested.

of the viruses appeared to be antigenically distinct. Frijoles was the only virus which was inhibited significantly by the heterologous immune reagents. An antigenic relationship was also demonstrated between RVF virus and four other PF group agents. RVF virus was neutralized by Candiru, Karimabad and Punta Toro immune sera, and RVF antiserum neutralized Frijoles virus.

Table 3 compares CF and PRN test results with three closely related phleboviruses (Naples, Tehran and Toscana). By CF test, the three agents were indistinguishable; but by PRN technique, they were antigenically distinct. On the basis of PRN test results, these viruses have been registered in the Arbovirus Catalogue as distinct agents (N. Karabatsos, Center for Disease Control, Ft. Collins, Colorado, personal communication, 1980).

Results of IFA tests are given in Table 4. Although some of the reactions were quite specific, others showed fairly broad cross-reactivity. At lower dilutions, RVF and Gordil antisera gave positive reactions with almost all of the antigens tested. Likewise, Itaporanga and Frijoles antigens reacted with most of the heterologous immune reagents. Several convalescent human sera from

TABLE 2
Continued

Virus type																
ICO	ITA	ITP	KAR	SFN	NIQ	PAC	PT	RVF	R(	SAF	SAL	SFS	TEH	TOS	TUA	URU
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	80	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	80	10	0	0	0	0	0	0	0	0
0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<20	<20	<20	<20	<20	<20	<20	<20	—	<20	<20	<20	<20	<20	<20	<20	<20
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	80	0	0	0	0	0	0
1,280	0	10	0	0	0	0	320	0	0	20	0	0	0	0	0	10
0	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	20	320	0	0	0	0	40	0	0	0	0	0	0	0	0
0	0	0	0	320	0	0	0	0	0	40	0	0	0	20	0	0
0	0	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	2,560	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	20	0	10,240	40	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	5,120	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	320	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	5,120	0	0	0	0	0	0
0	0	80	10	0	0	0	0	0	0	0	1,280	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	5,120	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	80	0	0	0
0	0	0	0	10	0	0	0	0	0	80	0	0	0	5,120	0	0
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	80	—
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	640

Egyptian patients infected with RVF virus were also examined by IFA; results with these specimens were similar to those obtained with the RVF rabbit immune serum (Table 4) and showed considerable cross-reactivity. These results indicate that the IFA is less specific than either the CF or PRN test but that it is similar to the HI test in revealing broad antigenic relationships among phleboviruses.

DISCUSSION

Results of PRN tests shown in Table 2 indicate that each of the 31 PF group viruses is antigenically distinct. Of the viruses tested, Frijoles was the only agent which was significantly neutralized by the heterotypic immune reagents. Still, there was no difficulty in distinguishing it from the other viruses.

The results of CF and PRN tests with Naples, Tehran and Toscana viruses (Table 3) gave quite different results. By CF test, the three agents were indistinguishable; however, by PRN technique, each of the viruses was distinct. These conflicting serologic results suggest that Naples, Tehran and Toscana viruses share a common antigen (probably the nucleocapsid antigen).

The prototype Naples strain was isolated from the serum of a sandfly fever patient in Italy in 1944.[12] *Tehran virus was recovered from Phlebotomus papatasi* collected in Iran in 1959.[13] Toscana virus was isolated from *Phlebotomus perniciosus* collected in Italy in 1971.[4] Initially, both Tehran and Toscana viruses were typed by CF test and were identified as Naples-like agents.[4, 13] Subsequent neutralization tests showed that they were different.

In addition to these three agents, there are other

TABLE 3

Results of complement-fixation (CF) and plaque reduction neutralization (PRN) tests with Naples, Toscana and Tehran viruses

Immune reagent	Antigen/virus					
	Naples		Toscana		Tehran	
	CF	PRN	CF	PRN	CF	PRN
Naples	256/128*	320†	128/256	40	128/64	<10
Toscana	64/64	20	256/≥512	≥5,120	64/64	<10
Tehran	128/128	<10	128/256	<10	256/128	80

* Reciprocal of highest serum dilution/highest antigen dilution.
† Reciprocal of highest serum dilution giving ≥90% plaque reduction.

viruses in the PF group which have given similar results. In studying viruses recovered from Panamanian sandflies, Tesh et al.[14] identified 50 isolates which showed varying degrees of cross-reactivity by CF test with both Buenaventura and Punta Toro viruses. The latter two agents are themselves closely related antigenically (Table 3).[3] Collectively, these observations suggest that considerable antigenic variation may occur by PRN test among PF viruses which are closely related or identical by CF test. Furthermore, this may be a general characteristic of phleboviruses. Our present knowledge of the PF serogroup is based on a relatively small sample of viruses. For many of the serotypes, only a single (prototype) strain has ever been isolated.[15] If antigenic diversity is a characteristic of viruses in this group, then the number of potential new serotypes, as defined by PRN test, may be almost limitless.

The observation of antigenic diversity among phleboviruses is of more than just academic interest, for it raises the question, "Does immunity to one member of the group provide cross-protection against infection with other closely related viruses?" In studies with human volunteers, Sabin demonstrated that persons infected with Naples or Sicilian virus did not become ill upon rechallenge with the homologous agent but were fully susceptible to infection with the heterologous virus.[12] Likewise, Bartelloni and Tesh found that the antibody response of volunteers infected with Sicilian virus was type-specific and that the patients' convalescent sera did not neutralize Naples, Arumowot or Salehabad viruses.[16] The

TABLE 4

Results of indirect fluorescent antibody (spot slide) tests with selected phlebotomus fever group viruses

Immune reagent	Antigen												
	RVF	PT	CDU	AMT	SFS	SFN	SAF	GOR	GF	ITP	ANH	FRI	NIQ
Rift Valley	2,048*	0	0	0	16	0	32	0	32	16	0	32	32
Punta Toro	64	2,048	0	8	0	0	8	0	64	32	0	16	128
Candiru	<8	0	256	0	0	0	0	0	0	0	0	0	16
Arumowot	8	0	0	256	0	0	0	0	0	0	0	4	0
Sicilian	<8	0	0	0	256	0	0	0	0	0	0	0	0
Naples	16	0	0	8	0	>1,024	128	4	0	16	0	32	16
Saint Floris	<8	0	0	0	0	0	>1,024	64	64	8	0	16	0
Gordil	32	16	8	4	4	32	64	>1,024	16	32	0	16	4
Gabek Forest	<8	0	8	0	0	0	4	0	>1,024	16	0	16	8
Itaporanga	8	0	0	0	0	0	0	0	0	256	0	16	0
Anhanga	32	8	8	0	0	0	0	0	0	0	512	16	0
Frijoles	64	0	0	0	4	0	16	0	0	0	0	>1,024	0
Icoaraci	32	32	0	0	0	0	8	0	16	32	0	32	16

* Reciprocal of highest dilution of immune reagent giving positive reaction. 0 = <1:4.

available data suggest that this may also be true of more closely related phleboviruses. Saidi et al.[17] found that the sera of persons with neutralizing antibodies against Naples virus did not neutralize Tehran virus. Likewise, Nicoletti et al.[9] found that human sera with antibodies to Toscana virus did not neutralize Naples virus. Results of these serologic studies confirm that Naples, Tehran and Toscana viruses are antigenically distinct and suggest that infection with one of these agents may not provide protection against the other two.

The apparent antigenic diversity among phleboviruses may be related to their segmented genome. Like other members of the family Bunyaviridae, the CF and neutralization determinants on the virion are thought to be coded for by different genes.[18] Thus, it is conceivable that some of these viruses may have similar nucleocapsid proteins but different glycoproteins (the presumed CF and neutralization determinants, respectively).[18] This might account for the extensive cross reactions observed with the CF test and the much greater specificity of the PRN test (Table 3). It is also possible that reassortment among closely related phleboviruses occurs in nature, further complicating their antigenic classification. This latter possibility seems plausible because (1) several PF virus serotypes are often active in the same locality simultaneously,[3, 19] (2) the field infection rate in sandflies with these agents is relatively high,[14, 18, 20] and (3) many of the sandfly-associated phleboviruses appear to be maintained in the vector population by transovarial transmission.[3, 14, 19, 20]

Results of the IFA test (Table 4) indicate that this procedure is less specific than either the PRN or CF tests for identification of phleboviruses. The degree of cross-reactivity observed with Itaporanga and Frijoles antigens and with RVF and Gordil antisera is comparable to that observed in HI tests.[3] Because of its broader cross-reactivity, the HI test has been the method of choice for identifying new members of the PF group. However, one problem in using the HI technique is the difficulty in preparing satisfactory hemagglutinins with some phleboviruses. The broadly reacting IFA test results suggest that this technique might be used as an alternative to the HI test for identifying new members of the PF group.

Initially it was hoped that the IFA test might be used as a rapid method for identifying specific *Phlebovirus* antibodies in human and animal sera. However, in view of the degree of cross-reactivity, this technique could only be used if each serum is titrated against all of the PF antigens which occur or might occur in a given geographic region. On the other hand, the IFA technique could be used as a screening procedure to detect PF group antibodies in areas where new or unknown serotypes are present.

ACKNOWLEDGMENT

We wish to thank Deborah Winograd for excellent technical assistance.

REFERENCES

1. Shope, R. E., Peters, C. J., and Walker, J. S., 1980. Serological relation between Rift Valley fever virus and viruses of phlebotomus fever serogroup. *Lancet, 1:* 886–887.
2. Rice, R. M., Erlick, B. J., Rosato, R. R., Eddy, G. A., and Mohanty, S. B., 1980. Biochemical characterization of Rift Valley fever virus. *Virology, 105:* 256–260.
3. Tesh, R. B., Peralta, P. H., Shope, R. E., Chaniotis, B. N., and Johnson, K. M., 1975. Antigenic relationships among phlebotomus fever group arboviruses and their implications for the epidemiology of sandfly fever. *Am. J. Trop. Med. Hyg., 24:* 135–144.
4. Verani, P., Lopes, M. C., Nicoletti, L., and Balducci, M., 1980. Studies on *Phlebotomus*-transmitted viruses in Italy: I. Isolation and characterization of a sandfly fever Naples-like virus. Pages 195–201 *in* J. Vesenjak-Hirjan et al., eds., *Arboviruses in the Mediterranean Countries*. Gustav Fischer Verlag, Stuttgart.
5. Peters, C. J., and Meegan, J. M., 1981. Rift Valley fever. *In* J. H. Steele, ed., *Handbook Series in Zoonoses,* Sect. B, *Viral Zoonoses,* Vol. 1. CRC Press, Boca Raton, Florida. In press.
6. Laughlin, L. W., Meegan, J. M., Strausbaugh, L. J., Morens, D. M., and Watten, R. H., 1979. Epidemic Rift Valley fever in Egypt: Observations of the spectrum of human illness. *Trans. R. Soc. Trop. Med. Hyg. 73:* 630–633.
7. Srihongse, S., and Johnson, C. M., 1974. Human infections with Chagres virus in Panama. *Am. J. Trop. Med. Hyg., 23:* 690–693.
8. Tesh, R. B., Saidi, S., Gadjdamovic, Ja. S., Rodhain, F., and Vesenjak-Hirjan, J., 1976. Serological studies on the epidemiology of sandfly fever in the Old World. *Bull. W.H.O., 54:* 663–674.
9. Nicoletti, L., Verani, P., Ciufolini, M. G., Lopes, M. C., and Zampetti, P., 1980. Studies on *Phlebotomus*-transmitted viruses in Italy: II. Serologic status of human beings. Pages 203–208 *in* J. Vesenjak-Hirjan et al., eds., *Arboviruses in the Mediterranean Countries*. Gustav Fischer Verlag, Stuttgart.
10. Casey, H. L., 1965. Standardized diagnostic com-

plement-fixation method and adaptation to micro-test. *Public Health Monograph No. 74*. U.S. Government Printing Office, Washington, D.C.

11. Riggs, J. L., 1979. Immunofluorescent staining. Pages 141–151 *in* E. H. Lennette and N. J. Schmidt, eds., *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*. American Public Health Association, Inc., Washington.
12. Sabin, A. B., 1951. Experimental studies on phlebotomus (Pappataci, Sandfly) fever during World War II. *Arch. Ges. Virusforsch., 4:* 367–410.
13. Barnett, H. C., and Suyemoto, W., 1961. Field studies on sandfly fever and kalaazar in Pakistan, in Iran, and in Baltistan (Little Tibet), Kashmir. *Trans. N.Y. Acad. Sci., 23:* 609–617.
14. Tesh, R. B., Chaniotis, B. N., Peralta, P. H., and Johnson, K. M., 1974. Ecology of viruses isolated from Panamanian phlebotomine sandflies. *Am. J. Trop. Med. Hyg., 23:* 258–269.
15. Berge, T. O., 1975. *International Catalogue of Arboviruses*. DHEW Publication No. (CDC) 75-8301. U.S. Department of Health, Education and Welfare, p. 789.
16. Bartelloni, P. J., and Tesh, R. B., 1976. Clinical and serologic responses of volunteers infected with phlebotomus fever virus (Sicilian type). *Am. J. Trop. Med. Hyg., 25:* 456–462.
17. Saidi, S., Tesh, R., Javadian, E., Sahabi, Z., and Nadim, A., 1977. Studies on the epidemiology of sandfly fever in Iran, II. The prevalence of human and animal infection with five phlebotomus fever virus serotypes in Isfahan Province. *Am. J. Trop. Med. Hyg., 26:* 288–293.
18. Bishop, D. H. L., and Shope, R. E., 1979. Bunyaviridae. Pages 1–156 *in* H. Fraenkel-Conrat and R. R. Wagner, eds., *Comprehensive Virology*, Vol. 14. Plenum Press, New York.
19. Tesh, R., Saidi, S., Javadian, E., and Nadim, A., 1977. Studies on the epidemiology of sandfly fever in Iran. I. Virus isolates obtained from *Phlebotomus*. *Am. J. Trop. Med. Hyg., 26:* 282–287.
20. Aitken, T. H. G., Woodall, J. P., Andrade, A. H. P., Bensabath, G., and Shope, R. E., 1975. Pacui virus, phlebotomine flies, and small mammals in Brazil: An epidemiological study. *Am. J. Trop. Med. Hyg., 24:* 358–368.